# PET methodology in rat models of Parkinson's disease

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# Chapter 8

Future Perspectives

Since its inception, positron emission tomography (PET) has been used extensively in neurological, cardiac and oncological disorders, because of its ability to non-invasively assess physiological processes. Nonetheless, new radioligands that are developed have to be evaluated for their suitability to quantify the corresponding target. Plasma input compartmental modeling is the gold standard for quantification of radioligands with PET imaging, but this quantification method requires a metabolite-corrected arterial input function and thus arterial blood sampling. Blood sampling is an invasive procedure in small animals like rats. Nevertheless, it is necessary especially for the comparison of non-invasive imaging quantification, i.e. methods without the need for plasma input, with the gold standard.

The first part of this thesis evaluated the quantification of two radioligands for the cholinergic system in rats. The radioligand [$^{11}$C]-PMP is an acetylcholine analog that is irreversibly hydrolyzed by acetylcholinesterase (AChE) and thus trapped in the human brain. The radioligand [$^{18}$F]-FEOBV is reversibly bound to the vesicular acetylcholine transporter (VAChT) in humans. Once specificity of radioligands for their target and the optimal quantification method are established, they can be used to quantify the expression or function of their target in patients or animal models of disease. In the second part of the thesis, PET imaging with [$^{18}$F]-FEOBV and radioligands for neuroinflammation and dopaminergic function were used in a toxin-based or transgenic model of Parkinson's disease.

One of the main findings of this thesis is further evidence for the need to validate radioligands for each species. The study described in Chapter 2 confirmed previous findings of the apparent reversibility of [$^{11}$C]-PMP trapping in rats, while the [$^{11}$C]-PMP metabolite is irreversibly trapped in humans [1]. Although we showed that quantification of [$^{11}$C]-PMP is possible using surrogate measures for the hydrolysis rate of [$^{11}$C]-PMP by AChE, further evaluation of the radioligand is necessary before it can be used in rats. Our and earlier studies [2, 3] suggest an active transport mechanism of the [$^{11}$C]-PMP metabolite over the blood-brain barrier which can hamper [$^{11}$C]-PMP quantification. Efflux transporters like P-glycoprotein (P-gp) have been shown to be more extensively expressed in rats than in humans [4]. Furthermore, efflux transporters could be affected in disease

models which, in turn, could bias the outcome of [$^{11}$C]-PMP quantification in an unknown manner. Hence, studies using efflux transporter inhibitors could be performed to evaluate their effect on [$^{11}$C]-PMP quantification. Additionally, our exploratory study did not include test-retest evaluation of [$^{11}$C]-PMP or the blocking of AChE to conclusively evaluate the cerebellum as a reference region for graphical analysis methods yielding the effective distribution volume (EDV) and the effective distribution volume ratio (EDVR) [5] or the standardized uptake volume ratio (SUVR). These issues should be addressed before [$^{11}$C]-PMP can be used in further studies in rats.

Compared to [$^{11}$C]-PMP, the kinetics of [$^{18}$F]-FEOBV did not vary drastically between humans and rats. Nevertheless, the radioligand still showed a different preferred quantification method in rats than in humans. We found the irreversible compartmental model and Patlak graphical analysis to fit the [$^{18}$F]-FEOBV data in rats best, whereas the reversible compartmental model was preferred in humans. Chapter 3 and 4 emphasize that no reference region for kinetic modeling in rats exists, while reference tissue approaches in humans were found to robustly quantify [$^{18}$F]-FEOBV binding to VAChT [6]. In humans, the cerebellar gray matter was used as reference region, while we assessed the cerebellum as a whole as reference tissue in rats. The smaller size of the rodent brain limits the brain regions that can be accurately delineated using PET imaging. Although the volumetric resolution of dedicated small animal PET scanners (approximately 2.2-3.4 mm$^3$) is better than that of clinically used PET scanners for humans (64-512 mm$^3$), the human brain is nearly 600-fold larger compared to the rat brain [4, 7]. Consequently, radioligands cannot be accurately quantified in smaller brain regions like the substantia nigra in rats, due to large partial-volume effects and poor signal-to-noise ratios in small regions of interest. Additionally, co-registration errors could influence the measures for reference tissues like the cerebellar gray matter thereby biasing reference tissue approaches. The use of radioligand-specific templates with co-registered atlases of regions of interest has been shown to improve the co-registration of radioligands [8] and was used in all chapters of this thesis. Therefore, co-registration errors are not likely to be responsible for the differences in the effects of the reference tissue between rats and humans.

Chapter 4 assesses [$^{18}$F]-FEOBV binding after $D_2$ receptor antagonism via raclopride and haloperidol pretreatment. While haloperidol is a $D_2$ receptor ligand it is also a potent $\sigma$ receptor ligand. The apparent decreases of cholinergic activity after haloperidol treatment in the cerebellum and other brain regions suggest off-target binding of [$^{18}$F]-FEOBV to $\sigma$ receptors. Thus, the differences in the results obtained when using a reference tissue for [$^{18}$F]-FEOBV between rats and humans might be related to the higher binding affinity of [$^{18}$F]-FEOBV to the rat $\sigma_1$ receptor compared to the human $\sigma_1$ receptor. While it has been suggested that [$^{18}$F]-FEOBV and other benzovesamicol-derived radioligands should not be used for VAChT PET imaging due to their off-target binding [9, 10], one could argue that they could still be used in diseases or disease models in which $\sigma$ receptors are not affected.

Interspecies differences as observed in Chapters 2 to 4 have been described for other radioligands. The most pronounced interspecies difference might be for radioligands which are P-gp substrates similar to [$^{11}$C]-PMP. As mentioned above efflux transporters like P-gp show higher expression in rats than humans, and Syvänen et al. showed large differences in the brain uptake of the radioligands [$^{11}$C]-Verapamil, [$^{11}$C]-GR205171, and [$^{18}$F]-Altanserin between rats and humans [11]. Furthermore, it was shown that [$^{11}$C]-PIB did not bind amyloid-$\beta$ in a transgenic mouse model of Alzheimer's disease due to structural differences in mouse and human amyloid-$\beta$ [12]. It should be noted that species differences between rodents can also occur. For example, (R)-N-(2-[$^{18}$F]fluoroethyl)-3-pyrrolidyl benzylate, a radioligand for muscarinic acetylcholine receptors, showed different brain retention patterns in mice and rats, due to the formation of different metabolites in the brain between both species [13].

Besides brain uptake, interspecies differences can also affect the peripheral metabolism of a radioligand. It was shown that 3-trans-FCWAY and 3-cis-FCWAY (radioligands for the serotonergic 5-$HT_{1A}$ receptor) are metabolized differently in rat, monkey and human hepatocytes [14]. Additionally, Walker et al. found that pretreatment with the inhibitors of the catechol-O-methyltransferase and the aromatic L-amino acid decarboxylase (AADC) is required to facilitate PET imaging of [$^{18}$F]-FDOPA in rats [15] while in humans, only AADC inhibition is necessary.

To perform compartmental modeling of radioligands, a metabolite-corrected arterial input function is necessary. Obtaining blood samples is more difficult in rodents than in humans. Although it is possible to obtain arterial blood samples from the rat's tail artery (Chapter 2, [15, 16]), the femoral artery is more commonly used [17]. The procedure for the exposure of the femoral artery is extensive and the animal is terminated after the procedure. Hence, it cannot be used for the test-retest assessment of radioligands. Nevertheless, it is possible to use a superficial branch of the femoral artery in the hind legs of rats to facilitate repeated blood sampling in the same rat [17]. The test-retest reliability of [$^{11}$C]-PMP has not yet been assessed and the test-retest reliability of [$^{18}$F]-FEOBV was assessed without blood sampling (Chapter 3). However, a future study could evaluate the test-retest reliability of both radioligands with plasma input models using the method described by Sijbesma et al. [17]. While this approach has been validated for the test-retest evaluation of radioligands, it has not been used in longitudinal animal disease models yet. Longitudinal PET assessment is often combined with behavioral tests and the surgical intervention in the hind legs could affect animal behavior. Therefore, the method for repeated blood sampling in rats needs to be evaluated for its effect on behavioral experiments before it can be used in longitudinal studies of animal disease models.

The total blood volume of rodents (approximately 1.5 ml in mice and 20 ml in rats) is much smaller than in humans (approximately 5000 ml). Hence, the blood samples need to be much smaller compared to humans which can lead to an larger error in the assessment of radioactivity or metabolites in blood and plasma, because of a lower radioactivity count rate of the samples especially for short-lived isotopes like $^{11}$C. However, the removal of a large proportion of blood can also lead to physiological changes which could affect radioligand pharmacokinetics and animal welfare in longitudinal studies [4]. Continuous blood sampling via a shunt can be used to decrease blood loss [18]. Nevertheless, as the radioactivity is determined in blood, a correction factor needs to be applied to determine radioactivity in plasma [19]. Hence, for the initial evaluation of new radioligands, manual blood sampling is still required. Furthermore, analysis of metabolites is still necessary although population-based metabolite-correction might be an option, depending on the radioligand and disease model. Taken together, blood sampling

cannot be avoided at least for the initial evaluation of new radioligands, and physiological changes should be considered when assessing the data.

The blood sampling approaches mentioned above require anesthetized animals. Anesthesia may lead to a confounding difference between clinical and preclinical PET imaging. The most common anesthetics used are inhalable agents, such as isoflurane, which are most convenient to apply to the animal. However, anesthesia can cause decreases in body temperature which can influence radioligand kinetics and metabolism, and thus quantification. While heating pads or lamps can be used to counteracted changes in body temperature, anesthesia can also influence brain metabolism, the binding of radioligands or even inhibit the release of neurotransmitters [20–23]. Some attempts have been made to image awake animals using restraining of the animal [24], motion tracking approaches [25, 26] or even a small PET scanner around the rat's head [27], but these approaches have their own disadvantages and are still not commonly used. Thus, the effects of anesthesia should be considered when analyzing PET data from animals and the time of anesthesia should be kept as short as possible.

Since it was shown that anesthesia decreases acetylcholine release in the synaptic cleft [28, 29], anesthesia is especially important to consider when evaluating radioligands targeting the cholinergic system like [$^{18}$F]-FEOBV or [$^{11}$C]-PMP in rats. As discussed in Chapter 4, our results obtained with [$^{18}$F]-FEOBV PET imaging after raclopride pretreatment are similar to results obtained in mice using ex vivo biodistribution with reduced time of anesthesia [30, 31]. Hence, it seems unlikely that the reduced acetylcholine release influenced the net influx rate measured with [$^{18}$F]-FEOBV. Similarly, it is unlikely that anesthesia by itself influenced [$^{11}$C]-PMP kinetics as studies in pigtail monekys anesthetized with ketamine showed the retention of the [$^{11}$C]-PMP metabolite in the brain [1, 32].

As outlined above there are certainly challenges associated with PET imaging in small animals, nonetheless, the advantages of PET imaging prevail. PET imaging with kinetic modeling compensates for differences in non-specific binding of radioligands or perfusion of tissue, which cannot be distinguished when using methods like ex-vivo biodistribution. Therefore, PET imaging offers the possibility

to accurately quantify physiological processes in vivo. Furthermore, it is possible to repeatedly assess the same animal (even with blood sampling) while other methods like biochemical assays or immunohistochemistry of brain tissue only provide information for a single time point. To evaluate several time-points with methods like immunohistochemistry cross-sectional study designs are required which can obscure intra-individual changes and require a larger number of animals.

The radioligands [$^{11}$C]-PMP and [$^{18}$F]-FEOBV have already been used to investigate a variety of research questions not only in Parkinson's disease, but also Alzheimer's disease patients. Parkinson's disease is associated with motor symptoms such as bradykinesia, tremor, and rigidity. However, non-motor symptoms like dementia, sleep problems and hallucinations are a significant additional burden for Parkinson's disease patients [33, 34]. First indications for an involvement of the cholinergic system came from postmortem studies showing reduced cholinergic innervation in the basal forebrain and reduced AChE activity in the frontal cortex of demented Parkinson's disease patients [35, 36]. Since then, several PET imaging studies using radioligands for the cholinergic system have found reduced AChE activity or expression of VAChT in demented Parkinson's disease patients [37–40]. Furthermore, studies using [$^{11}$C]-PMP found an association of reduced AChE activity in the cortex and thalamus with a history of falls in non-demented Parkinson's disease patients [41, 42], and an association of olfactory dysfunction with reduced AChE activity in the hippocampus, amygdala, and neocortex [43]. A study using [$^{18}$F]-FEOBV showed increased radioligand uptake in certain brainstem areas of idiopathic rapid eye movement sleep behavior disorder (RBD) patients compared to controls [44]. In Alzheimer's disease patients, [$^{18}$F]-FEOBV can be used for quantification of cholinergic denervation and was suggested as a potential biomarker for Alzheimer's disease [45].

As shown above the radioligands [$^{18}$F]-FEOBV and [$^{11}$C]-PMP can be used to evaluate cholinergic activity in several aspects of Parkinson's disease. In combination with animal models, the radioligands can help to elucidate the mechanisms leading to the development of cognitive impairment and other non-motor symptoms or assess the effectiveness of treatments on the cholinergic

activity in Parkinson's disease but also in other diseases. Studies in animal models of Parkinson's disease have several advantages compared to human studies. Animal models can be used to assess different aspects of disease development in a shorter time compared to human studies. They are cost-effective, short-lived and readily available. Compared to humans, they also show higher standardization, i.e. lower variation in genetic background and environmental influences. The possibility of genetic modification provides the opportunity to evaluate the effect of different genes involved in Parkinson's disease. An ideal disease model should show construct, face and predictive validity [46]. Construct validity represents the conceptual likeness between model and disease, e.g. similar genetic mutation, while face validity concerns the pathological features of the disease that the model embodies. The main pathological hallmarks of Parkinson's disease are the formation of Lewy bodies from α-synuclein and the progressive dopaminergic degeneration in the substantia nigra pars compacta, although the involvement of other neurotransmitters such as acetylcholine, noradrenaline or γ-aminobutyric acid (GABA) has also been shown [47, 48]. Additionally, animal models should replicate the motor and non-motor symptoms associated with Parkinson's disease. Lastly, treatment responses should be similar in human disease and in the disease model (predictive validity). For example, the 6-hydroxydopamine (6-OHDA) model in rats has been shown to be responsive to L-DOPA treatment [49].

While the numerous contributions of animal models to Parkinson's disease are widely recognized there is also agreement in the scientific community that there is no "perfect" model of Parkinson's disease as each model only characterizes a certain number of pathological features of the disease [50–53]. The most important limitation is that Parkinson's disease only develops spontaneously in humans and thus the disease always needs to be induced in animals. Another limitation is the lower susceptibility of rodents to toxins like 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), but also to inflammatory triggers such as lipopolysaccharide (LPS), compared to humans [50, 54]. Additionally, in humans, but not in rodents, neuromelanin is formed, e.g. in dopaminergic neurons in the substantia nigra or ventral tegmental. This has been shown to increase the vulnerability of neurons to neurodegeneration and might contribute to the lower

susceptibility of rodents to MPTP [50, 55, 56]. Many other differences between rodents and humans are known, including the aforementioned higher expression of efflux transporters, but also differences in gene regulation or behavior which can limit the ability to transfer of results found in animal studies to humans.

The limitations of each animal model should be considered before choosing a model for a specific research question. For example, the 6-OHDA model of Parkinson's disease has been characterized as an acute model of dopaminergic degeneration, which does not reflect the prolonged and progressive degeneration of dopaminergic neurons in humans [52]. Nevertheless, depending on the 6-OHDA injection site, the speed and extent of dopaminergic degeneration can differ. While the injection of 6-OHDA into the medial forebrain bundle or the substantia nigra leads to rapid degeneration of dopaminergic neurons [57], injection of 6-OHDA in the striatum, causes dopaminergic neurons to degenerate more slowly and retrogradely [58, 59]. Hence, it has been suggested that this model can be used for disease-modifying strategies. Therefore, we assessed the hypercholinergic state in Parkinson's disease and the influence of exercise on cholinergic activity in this 6-OHDA model. While exercise has shown mainly beneficial effects in Parkinson's disease [60–66] and healthy subjects [67–71], we could not replicate those findings in the 6-OHDA treated group or the control group. The study only showed small asymmetric changes in [$^{18}$F]-FEOBV uptake and no effect of 6-OHDA or exercise on contralateral forelimb use in the cylinder test. Thus, the reasons for this should be evaluated. As already discussed in Chapter 5, the cylinder test can lead to inconclusive results if dopaminergic denervation is mild [72]. Hence, immunohistochemistry with a marker of dopaminergic neurons should be performed to confirm the extend of degeneration of dopaminergic neurons. Additionally, exercise was performed during the light-phase for logistical reasons. This could have caused stress in the rats and thus confounded the beneficial effects of exercise. Additionally, the exercise was performed in the housing room of all rats in the study for practical reasons. It is possible that prolonged stress experienced by the rats in the exercise groups affected control rats housed in the same room [73]. In future studies, the exercise protocol should not be performed in the housing room to avoid elongated stress-responses. Furthermore, the possibility

to use voluntary instead of forced exercise should be explored as it could decrease stress in the rats [71].

Besides the evaluation of treatments, animal models can address research questions that cannot be appropriately evaluated in humans. Clinical diagnosis of Parkinson's disease usually occurs after the onset of motor symptoms, and approximately 50-60% of dopaminergic neurons have already degenerated at this time [74]. Although longitudinal clinical studies of prodromal hereditary Parkinson's disease or RBD patients – approximately 81% of which could develop Parkinson's disease later in life [44, 75, 76] – will shed new light on the development of Parkinson's disease, several years or even decades will pass before patients become symptomatic and the first conclusions can be drawn. Such studies are inherently limited by the patient cohort, having specific aspects of disease development that might not be present in sporadic Parkinson's disease. Contrarily, animal models can be widely used for the evaluation of risk factors of the disease including genetic causes.

Interestingly, most transgenic animal models have only shown limited replication of the pathological hallmarks of Parkinson's disease [50, 51], although more recent transgenic rat models seem to show higher face validity [53]. The age-dependent penetrance of some genes affected in Parkinson's disease, the relatively low number of hereditary Parkinson's disease cases and the link between environmental risk factors and Parkinson's disease suggest that it is a multi-factorial disease [77–79]. In human studies, environmental contributions like infections or food intake cannot be controlled. In contrast, animal studies offer the opportunity to assess environmental factors in a tightly controlled setting. We assessed the effect of single inflammatory trigger (LPS) on neuroinflammation and dopaminergic degeneration in a LRRK2 p.G2019S transgenic rat model using PET imaging, behavioral testing, and immunohistochemistry (Chapter 6). While no effect of LPS on dopaminergic innervation was found, we could show increased neuroinflammation in the cortical and ventral-brain region (average of brainstem and midbrain amongst others) 10 months after LPS treatment. The study was limited to the assessment of one neurotransmitter system over a period of one year. However, the involvement of neurotransmitters other than dopamine has

been shown in Parkinson's disease although their timing is unknown [47, 48]. As discussed in Chapter 6, the increased neuroinflammation in the cortex and a brain region encompassing midbrain and brainstem could have affected serotonergic, noradrenergic or cholinergic neurons, which were not evaluated in this study. In a recent study, Liu et al. showed increased AChE activity in LRRK2 mutation carriers without Parkinson's disease compared to healthy controls and in LRRK2 mutation carriers with Parkinson's disease compared to idiopathic Parkinson's disease and healthy controls amongst others in cortical brain region [80]. Hence, it would be of interest to evaluate the cholinergic system in the transgenic LRRK model described in Chapter 6.

The limited time assessed in the study described in Chapter 6 could have missed neuroinflammation in the striatum and substantia nigra at later times. Additionally, only a single intraperitoneal injection of LPS was performed in this study, whereas repeated infections usually occur in humans over a lifetime. Other studies have assessed the repeated application of low-dose LPS in rodent models of Parkinson's disease. For example, increased oxidative stress was found in the cortex in a 6-OHDA rat model treated repeatedly with LPS or control animals treated with repeated LPS-exposures alone [81]. Additionally, decreased spatial memory was observed in 6-OHDA rats repeatedly exposed to LPS compared to controls or rats treated with 6-OHDA alone. Furthermore, a study in mice found increased neuroinflammatory markers in the striatum and substantia nigra after repeated LPS exposure in combination with lactacystin, a proteasome inhibitor [82]. However, similar to our study no change in tyrosine hydroxylase-positive cells was found. Contrarily, repeated LPS-exposure before treatment with MPTP in mice aggravated dopamine depletion in the striatum and decreased stride-length compared to control groups [83]. These effects persisted until four months post-intervention. These findings indicate that repeated inflammatory triggers exacerbate the pathological features of various Parkinson's disease models. Although all studies above used 250 µg/kg LPS, the inflammatory trigger was applied in a relatively short period of time for three to seven consecutive days. Nevertheless, future studies can build on this foundation, and explore repeated LPS injections over a longer period of time, which would reflect infections in humans more closely.

Concluding, PET imaging is a valuable tool to assess protein expression or enzymatic function in vivo. We found that it is necessary to determine the optimal quantification method of radioligands in each species and fortunately this is already increasingly performed. The combination of PET imaging with Parkinson's disease models facilitates the assessment of numerous neurotransmitters or neuroinflammation in the same animal over long periods of time. Nevertheless, studies that expose animals to redundant stressors, like exercise during the light-phase or combining housing and experimental rooms, which can confound study outcomes in an unknown manner, should be avoided in the future. Nonetheless, animal models of Parkinson's disease in conjunction with methods like PET imaging, behavioral tests, biological assays, and immunohistochemistry, can aid in the evaluation of multiple aspects of the disease and thus increase our understanding of Parkinson's disease in ways which cannot be easily be implemented in human studies.

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